

Exhibit 1

Mechanism by Which Orally Administered β -1,3-Glucans Enhance the Tumoricidal Activity of Antitumor Monoclonal Antibodies in Murine Tumor Models¹

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Antitumor mAb bind to tumors and activate complement, coating tumors with iC3b. Intravenously administered yeast β -1,3;1,6-glucan functions as an adjuvant for antitumor mAb by priming the inactivated C3b (iC3b) receptors (CR3; CD11b/CD18) of circulating granulocytes, enabling CR3 to trigger cytotoxicity of iC3b-coated tumors. Recent data indicated that barley β -1,3;1,4-glucan given orally similarly potentiated the activity of antitumor mAb, leading to enhanced tumor regression and survival. This investigation showed that orally administered yeast β -1,3;1,6-glucan functioned similarly to barley β -1,3;1,4-glucan with antitumor mAb. With both oral β -1,3-glucans, a requirement for iC3b on tumors and CR3 on granulocytes was confirmed by demonstrating therapeutic failures in mice deficient in C3 or CR3. Barley and yeast β -1,3-glucan were labeled with fluorescein to track their oral uptake and processing in vivo. Orally administered β -1,3-glucans were taken up by macrophages that transported them to spleen, lymph nodes, and bone marrow. Within the bone marrow, the macrophages degraded the large β -1,3-glucans into smaller soluble β -1,3-glucan fragments that were taken up by the CR3 of marginated granulocytes. These granulocytes with CR3-bound β -1,3-glucan-fluorescein were shown to kill iC3b-opsonized tumor cells following their recruitment to a site of complement activation resembling a tumor coated with mAb. *The Journal of Immunology*, 2004, 173: 797–806.

Natural products useful in preventing or treating disease have been highly sought after throughout human history. A major problem in characterizing many natural products is that they represent a complex mixture of ingredients, each one of which may contribute to bioactivity. β -1,3;1,6-glucans from fungi (e.g., mushrooms) and yeast are well-known biologic response modifiers that function as immunostimulants against infectious diseases and cancer (1, 2). Unlike most other natural products, purified β -1,3-glucans retain their bioactivity. This has permitted the characterization of how β -1,3-glucans work on a cellular and molecular level.

Research with β -1,3;1,6-glucan from fungi, yeast, and seaweed, as well as β -1,3;1,4-glucan from barley, has shown that they function through stimulation of granulocytes (neutrophils and eosinophils), monocytes, macrophages, and NK cells. Two membrane β -1,3-glucan receptors that mediate biological responses to β -1,3-glucan have been characterized at a molecular level. The first to be reported was the inactivated C3b (iC3b)⁶ receptor known as CR3 (Mac-1, CD11b/CD18, or α _M β ₂ integrin) (3–5). CR3 is highly expressed on neutrophils, monocytes, and NK cells, whereas less is present on macrophages (6). Dectin-1 is the second β -1,3-glucan receptor to be described at a molecular level (7, 8). Dectin-1 is preferentially expressed on macrophages over granulocytes, while absent on NK cells (8). On macrophages, Dectin-1 is the dominant receptor mediating the phagocytosis of yeast (9), whereas CR3 performs this function with granulocytes (10, 11). Unique to CR3 are its two separate binding sites, one carbohydrate-binding site for β -1,3-glucan and a second site for the iC3b fragment of C3. The β -1,3-glucan-binding site is located within the C terminus of CD11b (4, 12), whereas iC3b-binding site maps to the N-terminal I-domain of the CD11b subunit of CR3 (13). Although the iC3b-binding function of CR3 allows it to bind avidly to iC3b-coated cells, CR3 is not triggered to mediate phagocytosis or cytotoxicity by ligation to cells bearing only iC3b. Activation of CR3 requires its dual ligation to both β -1,3-glucan and the iC3b deposited adjacent to the β -1,3-glucan on fungal cell walls by the complement system.

Mammalian cells do not produce β -1,3;1,6-glucans. Thus, iC3b-coated (opsonized) tumor cells are not killed by leukocytes via CR3. Nevertheless, CR3 mediates avid attachment of leukocytes to

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⁶ Abbreviations used in this paper: iC3b, inactivated C3b; CR3, complement receptor type 3, the iC3b receptor also known as Mac-1, CD11b/CD18, or α _M β ₂ integrin; WGP, whole glucan particle, particulate β -1,3-glucan derived from baker's yeast; NSG, neutral soluble glucan, a low-molecular-mass single-chain β -1,3-glucan derived from baker's yeast; WT, wild type; DTAF, fluorescein dichlorotriazine; F, fluorescein; BCF, fluorescein-labeled barley β -1,3-glucan; WGP-F, fluorescein-labeled WGP.

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iC3b-coated tumor cells. An important finding was that soluble yeast β -1,3;1,6-glucan, as well as soluble barley β -1,3;1,4-glucan, bound to CR3 and primed the receptor to mediate leukocyte cytotoxicity of tumor cells coated with iC3b (5, 14). Cytotoxicity was blocked by anti-CR3 and did not occur with leukocytes from CR3-deficient mice. Moreover, β -1,3-glucan promoted this activity with human and mouse NK cells that express CR3 (5, 14, 15) but not Dectin-1 (8).

Intravenous injection of soluble yeast β -1,3-glucan into mice with mammary tumors resulted in regression of tumor growth. Tumor regression required natural antitumor Abs that bound to the tumors and activated complement with deposition of iC3b on the tumor cells. Therapy failed in SCID mice, which are missing B and T lymphocytes, but could be reconstituted by injection of natural Abs isolated from normal mouse serum. Therapy failures in C3- and CR3-deficient mice confirmed the requirement for iC3b on tumors and CR3 on leukocytes (16). Later studies showed that therapeutic efficacy could be enhanced significantly by injecting mice with complement-activating antitumor mAb that greatly increased the amount of tumor-bound iC3b. As expected, the efficacy of combining i.v. β -1,3-glucan with mAb was not observed in mice deficient in either C3 or CR3 (17).

In addition to these findings with i.v. β -1,3-glucan, there were also earlier reports that some mushroom β -1,3;1,6-glucans could mediate tumor regression when given orally (18, 19). In more recent studies using human tumor xenografts, orally administered soluble barley β -1,3;1,4-glucan or i.v. antitumor mAb were ineffective as single agents, but, when combined, elicited a substantial antitumor effect (20, 21). However, the mechanism by which large β -1,3-glucans could be taken up orally by the gastrointestinal tract and function to prime leukocyte CR3 was unknown.

The current investigation examined the mechanism of oral uptake of soluble barley and particulate yeast β -1,3-glucan and showed that these large β -1,3-glucans were taken up by gastrointestinal macrophages and shuttled to reticuloendothelial tissues and bone marrow. Within the marrow, the macrophages degraded the β -1,3-glucan and secreted small soluble biologically active fragments that bound to CR3 of mature bone marrow granulocytes. Once recruited from the bone marrow by an inflammatory stimulus, these granulocytes with β -1,3-glucan-primed CR3 could kill iC3b-coated tumor cells. As had been found earlier with i.v. soluble yeast β -1,3;1,6-glucan therapy, oral β -1,3-glucan-mediated tumor regression required the presence of iC3b on tumors and CR3 on granulocytes, and therefore failed in mice deficient in C3 or CR3.

Materials and Methods

Abs and other reagents

The hybridoma producing 14.G2a (IgG2a) anti-G₀₂ mAb (22), was generously provided by Dr. R. Reisfeld (Research Institute of Scripps Clinic, La Jolla, CA). The BCP8 hybridoma producing IgG2b anti-human MUC1 mAb was previously described and is specific for the protein neopeptide revealed in underglycosylated MUC1 (23). The B5 hybridoma secreting mouse IgG2a mAb specific for the human high-molecular-mass melanoma Ag was obtained from the American Type Culture Collection (Manassas, VA) and used as a nonspecific mAb control for mouse tumor therapy protocols. Each hybridoma was grown in BioReactor flasks (BD Biosciences, San Jose, CA) from which the mAb were purified as described previously (16, 17). Rat RB6-8C5 and mouse Gr-1-PE (anti-Gr-1-PE) and a PE-labeled isotype control were purchased from BD Pharmingen (San Diego, CA). Rat anti-mouse macrophage P4/80-PE-Cy5.5, as well as a PE-Cy5.5-labeled isotype control, were purchased from Caltag Laboratories (Burlingame, CA).

Therapeutic β -1,3-glucans

Whole glucan particles (WGP; Biopolymer Engineering, Eagan, MN) were purified from baker's yeast through a series of alkaline and acid extractions to yield hollow yeast cell wall ghosts composed primarily of β -1,3;1,6-glucan (24). WGP were hydrated by addition to distilled water and sonicated to produce a single-particle suspension. The soluble yeast β -1,3;1,6-glucan known as neutral soluble glucan (NSG) β -1,3-glucan (Biopolymer Engineering) and its ability to bind to and prime CR3 were previously described (4, 5, 14, 17). Highly purified large-molecular-size barley β -1,3;1,4-glucan was prepared and characterized as previously described (21). Barley β -1,3-glucan was completely dissolved by boiling for 10 min in normal saline.

Mice and tumor models

The murine tumor therapy protocols were performed in compliance with all relevant laws and institutional guidelines, and were approved by the Institutional Animal Care and Use Committee of the University of Louisville. Normal C57BL/6 mice were purchased from National Cancer Institute (Frederick, MD). Colonies of C57BL/6 mice deficient in either C3 or CR3 (CD11b^{-/-}) and their wild-type (WT) littermates were generated and maintained for tumor therapy protocols as previously described (17). RMA-S-MUC1, a C57BL/6 lymphoma cell line transfected with human MUC1 and its use in tumor therapy protocols in combination with 14.G2a anti-G₀₂ ganglioside and β -1,3-glucan, were previously described (17).

C57BL/6 WT vs CR3-deficient mice were examined using the RMA-S-MUC1 tumor protocol with oral barley or WGP β -1,3-glucan in 16 groups of six mice. For this protocol, one group of WT and one group of CR3-deficient mice were given oral PBS as a control for the oral β -1,3-glucans in water or saline. Two groups of WT or CR3-deficient mice received only the 14.G2a anti-G₀₂ ganglioside mAb, 100 μ g per i.v. dose given every 3 days. Four groups of mice (two WT and two CR3-deficient) received either oral barley or oral WGP β -1,3-glucan, 400 μ g per dose every day, beginning on the same day as the mAb therapy (preliminary experiments had shown that maximum tumor regression was obtained with a 400- μ g daily dose of either barley or yeast WGP β -1,3-glucan). The final eight groups of mice received a combination of i.v. 14.G2a mAb every third day and either oral barley or oral WGP β -1,3-glucan given daily, with the oral β -1,3-glucan administration beginning either on the same day that the i.v. mAb was administered or 3 days before administering the mAb. Therapy was continued for 3 wk, with tumor diameter measurements made with calipers every third day, and mice were sacrificed if tumors reached 15 mm in diameter. Mice were observed for tumor-free survival over 90 days.

The generation of a Lewis lung carcinoma cell line transfected with human MUC1 (LL/2-MUC1) and their use in tumor therapy protocols with BCP8 anti-MUC1 and β -1,3-glucan were previously described (17). Six groups of six C3-deficient mice or their WT littermates (total of 12 groups of mice) were treated with the following: 1) i.v. PBS every third day (control); 2) 100 μ l of barley or yeast WGP β -1,3-glucan (4 mg/ml in saline or water; total, 400 μ g) given daily using an intragastric gavage needle and syringe; 3) 200 μ g of BCP8 anti-MUC1 mAb given i.v. every third day; 4) combined therapy with BCP8 mAb and oral barley or WGP β -1,3-glucan. Therapy was given for 3 wk with measurement of tumor diameters every third day, and mice were sacrificed when tumors reached 15 mm in diameter. Mice were observed over a period of 100 days for tumor-free survival.

Analysis of the fate of orally ingested barley and yeast WGP β -1,3-glucan

Barley and yeast WGP β -1,3-glucan were labeled with fluorescein using fluorescein dichlorotriazine (DTAF; Molecular Probes, Eugene, OR), which covalently reacts with hydroxyl groups of polysaccharides using a modification of the procedures suggested by the manufacturer. Briefly, 50 mg of barley β -1,3-glucan was diluted to 20 mg/ml with borate buffer (pH 10.8), and mixed with 10 mg of DTAF dissolved in 0.5 ml of DMSO and heated for 1 h at 75°C. This generated a barley β -1,3-glucan gel that was pelleted by centrifugation and dissolved in 5 ml of 200-mM NaOH at 75°C. The DTAF-labeled barley β -1,3-glucan (BG-F) was then precipitated with ethanol. The pellet was dissolved in 200 mM NaOH at 75°C, and the process of precipitation followed by solubilization was repeated several times until a clear supernatant free of unbound DTAF was obtained. After final solubilization in a small volume of NaOH, the BG-F was diluted in PBS to a concentration of 4 mg/ml, and the pH was adjusted to 7.2.

DTAF at 2 mg/ml was added to a suspension of yeast WGP β -1,3-glucan (20 mg/ml) in borate buffer (pH 10.8), and incubated at room temperature overnight with continuous stirring. Unbound DTAF was removed by centrifugation and washing the pelleted fluorescein-labeled WGP

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(WGP-F) several times with PBS. To remove any traces of LPS contamination, the WGP-F was suspended in 200 mM NaOH for 20 min at room temperature and washed several times in LPS-free water and finally in LPS-free PBS. The concentration of the WGP-F was adjusted to 4 mg/ml in PBS for storage at room temperature.

Groups of WT or CR3-deficient C57BL/6 mice were given the WGP-F or BG-F in daily oral doses of 400 μ g. Beginning on day 1 and continuing daily for up to 10 days after daily administration of oral β -1,3-glucan-fluorescein (F), groups of three mice were sacrificed, and cell suspensions were made from isolated spleen, peritoneal lymph nodes, and bone marrow from tibia and femurs. The cell suspensions were analyzed for β -1,3-glucan-F by fluorescence microscopy with an automated digital camera. In some experiments, macrophages in spleen and bone marrow suspensions were enriched by absorption and EDTA elution from fibronectin-coated culture plates (BD Biosciences). In other experiments where noted, macrophages were identified in cell suspensions by staining with F4/80-PE-Cy5.5.

Analysis of in vitro macrophage degradation of ingested yeast WGP β -1,3-glucan

The murine macrophage line J774 (from the American Type Culture Collection) was plated at 1×10^5 cells per well of six-well plates in DMEM with 10% FCS, penicillin-streptomycin, and glutamine, and after 16 h of culture, were incubated with WGP-F at a 10:1 particle-to-cell ratio at 37°C. Cultures without added WGP-F served as controls. Using fluorescence microscopy, the phagocytic index for WGP-F was estimated by counting the proportion of cells that had internalized one or more WGP-F particles after 1 h. Typical phagocytic indices of >80% were observed. Macrophage cultures with ingested WGP-F were maintained for up to 21 days, and culture medium was collected daily and frozen for β -1,3-glucan analysis and fluorescence measurements. Periodically, cultures were examined by fluorescence microscopy for evaluation of the presence and size of internalized WGP-F. Culture supernatants and cell lysates were evaluated for total and soluble (10,000 \times g; 10 min) fluorescent and biologically active β -1,3-glucan levels (GlucateLL Bios assay; Associates of Cape Cod, Falmouth, MA). Soluble β -1,3-glucan-containing culture supernatants from days 6–14 were pooled and fractionated using Nanosep 3K nominal molecular mass cutoff centrifugal filters (Pall Corporation, East Hills, NY), and retentate and filtrate samples were assayed for fluorescence and β -1,3-glucan levels as described above.

To generate soluble β -glucan from macrophage-degraded WGP for analysis of bioactivity, J774 cells were cultivated on collagen-coated beads (Solo Hill Engineering, Ann Arbor, MI) in the same two-chamber Integra BioReactor flasks used for mAb production (BD Biosciences). After loading the J774 cell-coated beads into the lower chamber in 20 ml of medium with 10% FCS, 1.0 ml of a 4 mg/ml suspension of WGP in medium was added to the lower chamber and mixed with the beads by gentle pipetting. Microscopic visualization of the beads showed a confluent coating of J774 cells, each apparently filled to capacity with ingested WGP. After 1 wk, the spent medium in the top chamber (without FCS) was decanted and replaced, and the cell supernatant in the lower chamber (containing all detectable soluble β -1,3-glucan) was removed with a 25-ml pipette after mixing gently and allowing the beads to settle. The flask was returned to the incubator after adding 20 ml of fresh medium with 10% FCS to the lower chamber. Similar harvests of the lower chambers were conducted at weekly intervals for a total of 3 wk, after which time the J774 cells released no additional glucan, and the cell viability was too low to consider additional cultivation with freshly added WGP. The medium from the lower chamber cell harvests was dialyzed two times via 4 liters of PBS, concentrated with a 10-kDa nominal molecular mass cutoff membrane (Centricon; Millipore, Milford, MA), and hexose measurements were made to estimate β -1,3-glucan concentration.

Analysis of elicited peritoneal granulocytes for surface-bound β -1,3-glucan

Two groups of 10 WT mice and two groups of 10 CR3-deficient mice were given a daily oral dose of 400 μ g of BG-F or WGP-F for a total of 10 days. Peritoneal granulocytes from each mouse were separately analyzed 4 h after the i.p. injection of 5 ml of Brewer's thioglycoline medium (BD Diagnostic Systems, Sparks, MD). The mice were sacrificed by CO₂ asphyxiation, and peritoneal washes were collected by three washes of the surgically exposed peritoneal cavity with 2 ml of ice-cold PBS. Granulocytes in the peritoneal washes were stained at 0°C with anti-Gr-1-PE, and then the presence of β -1,3-glucan-F on Gr-1-PE⁺ granulocytes was determined by two-color flow cytometry analysis. Peritoneal cells obtained from mice that had not been given oral β -1,3-glucan-F served as a negative control for β -1,3-glucan-F staining.

Analysis of peritoneal granulocyte-mediated cytotoxicity

RMA-S-MUC1 cells were coated with iC3b by the sequential addition of BCP8 IgG2b anti-MUC1 mAb and fresh mouse serum as a source of complement (5). Peritoneal granulocytes were isolated as above from WT or CR3^{-/-} mice that had been given oral yeast WGP β -1,3-glucan for 10 days. Granulocytes from mice that had not received oral β -1,3-glucan served as a control. To determine whether granulocytes were fully primed with surface-bound β -1,3-glucan in vivo, soluble NSG β -1,3-glucan (10 μ g/ml) was added to some of the assay wells. The only preparation of NSG β -1,3-glucan that was available had a lower affinity for CR3 than previously reported NSG β -1,3-glucan preparations (4), and this resulted in a relatively low maximum level of cytotoxicity and a requirement for 10 μ g/ml NSG β -1,3-glucan rather than the 1.0 μ g/ml concentration required previously for high-affinity NSG β -1,3-glucan preparations (14). Tumor cells coated with iC3b were labeled with ⁵¹Cr and used as targets for peritoneal granulocyte cytotoxicity through measurement of the release of target cell ⁵¹Cr, using methods previously described with either human or mouse leukocytes (5, 14). Assays were conducted in triplicate using 96-well plates, and mean values were used for calculations of cytotoxicity. Granulocyte-to-tumor cell ratios were tested over the range of 3:1 to 50:1 to determine the ratio that produced optimal glucan-specific cytotoxicity. Soluble β -1,3-glucan isolated from J774 macrophages cultured with WGP in BioReactor flasks (see above) was similarly tested with the same cytotoxicity assay using peritoneal granulocytes isolated from WT vs CR3^{-/-} mice.

Analysis of splenic macrophage-mediated cytotoxicity

Mice were fed 400 μ g of barley β -1,3-glucan daily by intragastric gavage and sacrificed on sequential days for isolation of splenic macrophages prepared by finely mincing the spleens and passing the cells through a 70- μ m strainer. Macrophages obtained from mice not given barley β -1,3-glucan served as a control for the effect of in vivo exposure to β -1,3-glucan. PTAS-64 murine mammary adenocarcinoma cells (17) were labeled with ⁵¹Cr at a ratio of 100 μ Ci per 10⁶ cells and opsonized with 11C1 anti-MMTV mAb (17) and fresh mouse serum as a source of complement to coat the cells with iC3b (5). Macrophages and iC3b tumor cells were incubated in 96-well plates at E:T ratios ranging from 5:1 to 40:1 tested in triplicate for 4 h at 37°C. To demonstrate the requirement for CR3 in tumor killing, some samples were treated with M170 rat anti-mouse CR3 mAb (5).

Graphing and statistical analysis of data

Data from mouse therapy protocols were entered into Prism 4.0 (GraphPad Software, San Diego, CA) to generate graphs of tumor regression or survival and to determine the significance of differences between data sets. Student's *t* test was used to compare differences between two tumor regression curves, whereas the log rank test was used to determine the significance of differences between two survival curves.

Results

The antitumor activity mediated by oral β -1,3-glucans requires C3 and CR3

Recent reports that examined tumor xenograft models had shown that orally administered barley β -1,3;1,4-glucan (20, 21) functioned in the same way as did i.v. soluble yeast β -1,3;1,6-glucan tested with syngeneic tumor models (17), with both routes of β -1,3-glucan administration enhancing the tumor regression activity of complement-activating antitumor mAb. Additional experiments showed that yeast β -1,3;1,6-glucan particles (WGP) given orally also potentiated antitumor mAb therapy.

A similar requirement for complement in the tumor regression mediated by oral barley and yeast i.v. β -1,3-glucan had been suggested because tumor regression required use of complement-activating antitumor mAb (16, 17, 20), and therapy with an IgG1 mAb that did not activate complement was not enhanced by oral barley β -1,3-glucan (21). To determine whether oral β -1,3-glucan therapy had the same requirement for iC3b on tumors and CR3 on leukocytes as had been shown with i.v. yeast β -1,3-glucan therapy (16, 17), tumor therapy protocols were conducted comparing WT C57BL/6 mice to either CR3-deficient (CD11b^{-/-}) or C3-deficient (C3^{-/-}) C57BL/6 mice (Figs. 1–4).

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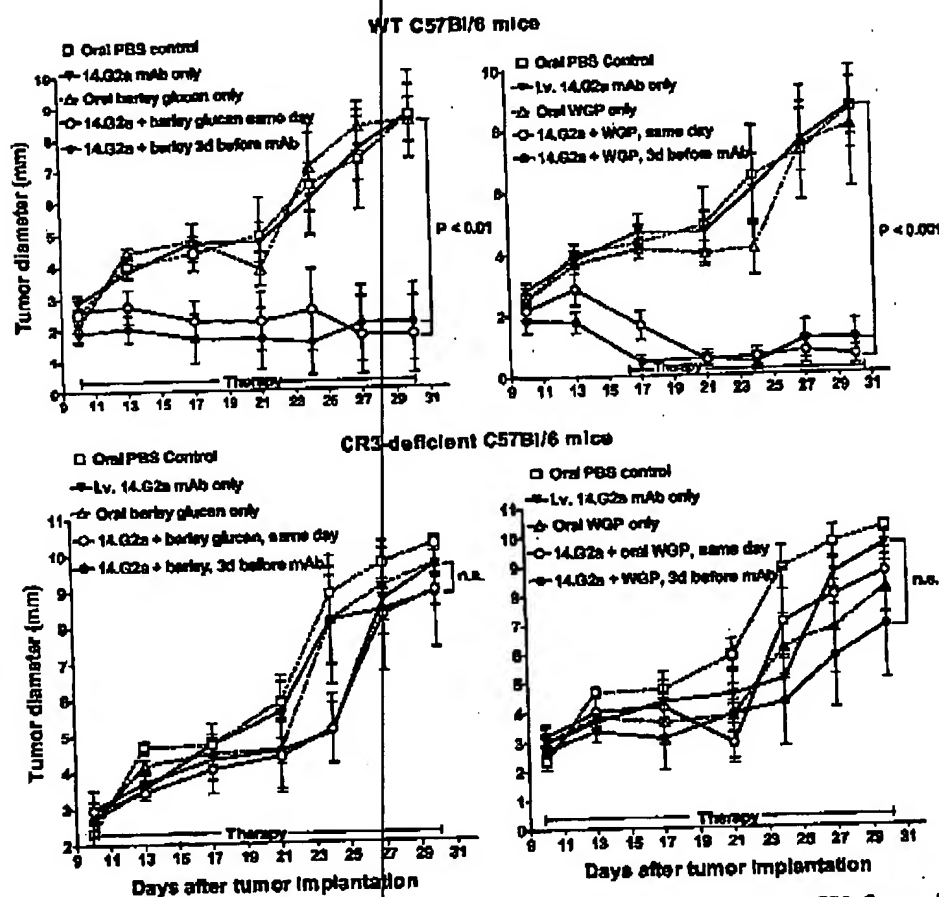
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FIGURE 1. Tumor regression with antitumor mAb plus oral barley or yeast WGP β -1,3-glucan requires leukocyte CR3. Groups of WT or CR3-deficient C57BL/6 mice were implanted s.c. with RMA-S-MUC1, and after 10 days, to allow tumor formation, were treated with i.v. 14.G2a anti-G_{D2} ganglioside with or without simultaneous oral barley or yeast WGP β -1,3-glucan. Some groups of mice were given the oral β -1,3-glucan on the same day as the i.v. mAb (day 10), whereas other groups of mice were given the oral β -1,3-glucan 3 days before the mAb (day 7). Mean values \pm SD are shown.

The protocol used to compare WT to CR3-deficient mice used RMA-S-MUC1 tumor cells and 14.G2a anti-G_{D2} ganglioside mAb (Figs. 1 and 2). Groups of mice were given either oral barley or yeast WGP β -1,3-glucan on the same day as the mAb or 3 days before the mAb. With both β -1,3-glucans, earlier oral administration gave more rapid tumor regression, and therefore this early administration of oral β -1,3-glucan was used in subsequent protocols. However, after 3 wk of therapy, there was no difference in the regression resulting from giving the β -1,3-glucan at the earlier time point (Fig. 1). Treatment with mAb alone elicited no tumor regression, whereas combining the i.v. mAb with oral barley or yeast WGP β -1,3-glucan elicited significant regression in WT but not in CR3-deficient mice. Moreover, the combined treatment with i.v. mAb and oral β -1,3-glucans produced 60–100% survival in WT mice, but only 0–20% survival in the CR3-deficient mice (Fig. 2). The tumor regression in mice treated only with β -1,3-glucan was likely due to naturally occurring antitumor Abs, because no elicited anti-MUC1 was detectable in the sera of mice harboring these tumors. These experiments demonstrated a near-absolute requirement for leukocyte CR3 for the antitumor effect, especially when oral barley β -1,3-glucan was given with antitumor mAb.

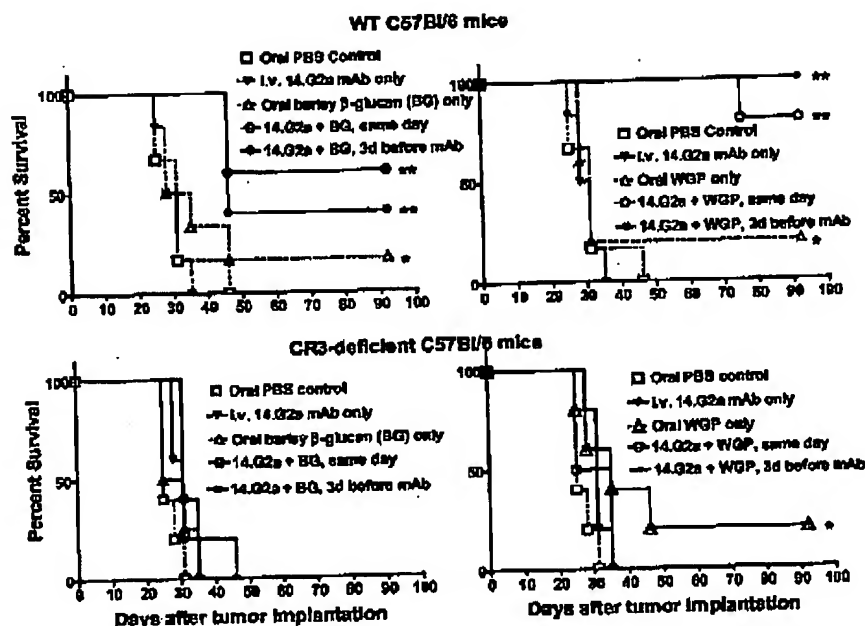
A protocol comparing WT to CR3-deficient mice similarly showed that oral β -1,3-glucan therapy required serum C3 (Figs. 3 and 4). Mice bearing s.c. Lewis lung carcinoma cells transduced

with human MUC1 (LL/2-MUC1) were treated with BCP8 (1gG2b) anti-MUC1 mAb. Treatment with oral barley or yeast WGP β -1,3-glucan and i.v. mAb elicited significantly enhanced tumor regression compared with that mediated by mAb alone. A small antitumor effect observed in mice treated with β -1,3-glucan alone was likely due to naturally occurring Ab to the parent LL/2 cells. By contrast, neither β -1,3-glucan given orally alone or in combination with mAb could elicit tumor regression in CR3-deficient mice. In addition, both oral β -1,3-glucans, when combined with mAb, achieved 40% survival in WT mice vs no survival in CR3-deficient mice (Fig. 5). Examination of tumors from mice that escaped the combined mAb plus β -1,3-glucan therapy showed that >75% of the tumor cells no longer expressed the MUC1 target Ag.

Oral β -1,3-glucans are ingested by macrophages that transport them to lymphoid organs

To determine the mechanism for oral uptake of β -1,3-glucan, both barley β -1,3-glucan and yeast WGP β -1,3-glucan were labeled with fluorescein (BG-F and WGP-F) and given to mice by intragastric injection in a manner similar to tumor therapy protocols. With the barley β -1,3-glucan, fluorescence microscopy revealed the generation of microaggregates by the DTAF fluorescein-labeling procedure. Groups of mice were sacrificed periodically and examined for BG-F and WGP-F in lymph nodes, spleen, and bone

FIGURE 2. Long-term survival following treatment with antitumor mAb plus oral barley or yeast WGP β -1,3-glucan requires leukocyte CR3. This is the survival analysis of the experiment described in Fig. 1. In WT mice, both the barley and yeast WGP β -1,3-glucans given in combination with mAb produced significantly better survival than did the therapy with mAb alone (**, $p < 0.01$). In CR3-deficient mice, therapy with mAb plus yeast WGP β -1,3-glucan produced significantly better survival than did any of the other therapy groups (*, $p < 0.05$).



marrow. Within 3 days of daily oral administration of BG-F or WGP-F, macrophages in the spleen (Fig. 5, *A*, *B*, and *H*) and lymph nodes (not shown) contained fluorescein-labeled β -1,3-glucan. After 4 days, WGP-F and BG-F were also observed in bone marrow, and double-staining for macrophages with F4/80-PE-Cy5.5 (red surface stain) confirmed that the cells containing BG-F or WGP-F were macrophages (Fig. 5, *E*, *F*, and *I*). In comparing the uptake of WGP-F and BG-F by WT vs CR3-deficient mice, no differences were apparent in either the percentage of macrophages containing ingested β -1,3-glucan-F or the amount of β -1,3-glucan-F per cell (Fig. 5, compare *C*, WT bone marrow macrophages, to *D*, bone marrow macrophages from CR3-deficient mice). Thus, the uptake of barley and yeast β -1,3-glucan by gastrointestinal macrophages does not require CR3 and is likely mediated instead by Dectin-1 (7, 25).

Macrophages secrete β -1,3-glucan fragments that bind to granulocyte CR3

After administering oral β -1,3-glucan for 3 days, the WGP-F and BG-F within splenic macrophages appeared to be the same size as the starting material (Fig. 5, *B* and *I*). However, macrophages isolated from mice given oral β -1,3-glucan-F for a longer period of time appeared to have begun breaking down the particles and aggregates into smaller fragments of β -1,3-glucan-F that were concentrated at the edges of the cytoplasm near the membrane (Fig. 5, *C* and *D*). When macrophages containing the degraded green β -1,3-glucan-F were surface stained with red F4/80-PE-Cy5.5, some of the green β -1,3-glucan-F appeared to be localized to the cell surface, because an overlay of the red and green staining showed areas of orange surface staining (Fig. 5*F*).

These experiments suggested that macrophages were able to partially degrade the large molecules of both soluble barley β -1,3-glucan and particulate yeast β -1,3-glucan. Studies conducted with cultures of the macrophage cell line J774 examined the fate of WGP-F added to the cultures (Fig. 6). These experiments showed that ingested WGP-F were slowly degraded within J774 macrophages and that soluble biologically active fragments of β -1,3-glucan-F were released into the culture medium that could be mea-

sured using a β -1,3-glucan-specific bioassay. Complete macrophage degradation of all visible cytoplasmic β -1,3-glucan-F required >13 days. Typically ingested WGP-F particles remained intact for 3–5 days, appeared to fragment into smaller particles and soluble material (5–10 days), and the intracellular fluorescence disappeared after 14–21 days. During this time, there was complete concordance in the amounts of biologically active β -1,3-glucan and fluorescein in culture supernatants. Moreover, the β -1,3-glucan bioactivity and fluorescein were retained during concentration of the supernatants with a 3-kDa molecular mass cutoff membrane that would have allowed the passage of small β -1,3-glucan-F oligosaccharides or unbound fluorescein. These data indicated that macrophages degrade large molecules of barley or yeast β -1,3-glucan into smaller biologically active fragments of β -1,3-glucan that are released into the medium.

Although all the bone marrow cells containing ingested β -1,3-glucan-F were initially shown to be macrophages identified by F4/80-PE-Cy5.5 staining (Fig. 5, *E*, *F*, *H*, and *I*), nonmacrophage (F4/80-negative) cells bearing membrane fluorescein staining began to appear in the bone marrow by day 5 of oral yeast WGP-F administration (7). Morphologically, these cells appeared to be granulocytes, and they were observed only in the bone marrow of WT and not CR3-deficient mice. To determine whether the soluble β -1,3-glucan-F released by macrophages had indeed been taken up by bone marrow granulocytes, groups of WT or CR3-deficient mice that had been given WGP-F or BG-F for 10 days were injected i.p. with thioglycolate medium to elicit the marginated pool of bone marrow granulocytes into the peritoneal cavity. Although the majority of peritoneal cells recruited by 4-h thioglycolate treatment are granulocytes, the elicited cells were stained with anti-Gr-1-PE to confirm their identification as granulocytes (Fig. 7). Granulocytes elicited from mice that had not been administered WGP-F or BG-F served as negative controls for fluorescein staining. This experiment detected significantly more membrane-bound fluorescein on WT than on CR3-deficient granulocytes. The enhanced granulocyte staining of cells bearing CR3, along with the finding that the fluorescein released from cultured macrophages corresponded to soluble β -1,3-glucan, indicated that the fluorescein

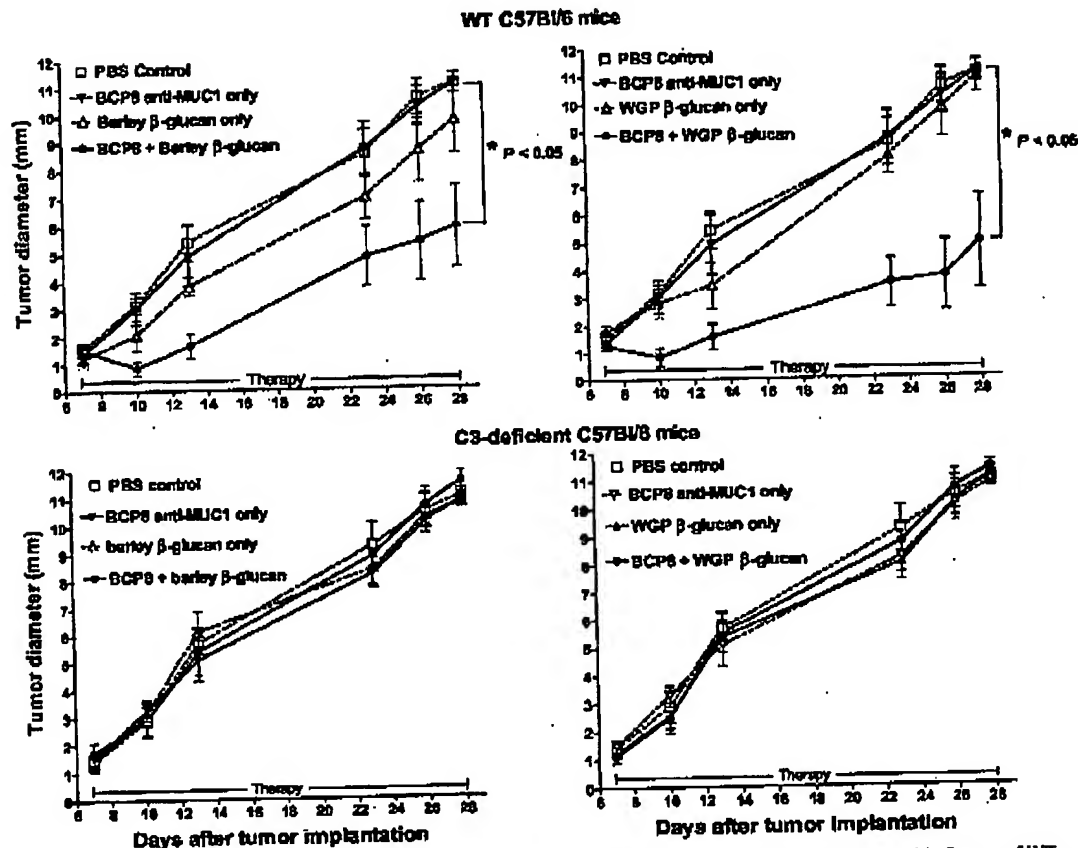
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FIGURE 3. Tumor regression with orally administered soluble barley or particulate yeast β -1,3-glucan requires serum C3. Groups of WT or C3-deficient C57BL/6 mice were implanted s.c. with LL2-MUC1 and after 7 days were treated with i.v. BCP8 anti-MUC1 with or without simultaneous oral barley or yeast β -1,3-glucan (WGP). Mean values \pm SD are shown.

staining represented membrane-bound β -1,3-glucan-F, which was predominantly attached to CR3. This conclusion is supported by previous research showing that soluble β -1,3-glucan-F added to peritoneal neutrophils *in vitro* was bound to the surface of WT but not CR3^{-/-} neutrophils (5). Thus, these data support a mechanism of oral β -1,3-glucan activity involving the sequential ingestion of β -1,3-glucan by gastrointestinal macrophages that shuttle the β -1,3-glucan to the bone marrow where soluble degradation fragments are released and taken up by granulocytes via membrane CR3.

Granulocytes with CR3-bound β -1,3-glucan are able to kill iC3b-coated tumors

Tumors that activate complement via the binding of antitumor Abs are thought to become inflammatory foci through the release of the chemotactic factor C5a that functions to recruit the marginated pool of granulocytes, as well as tissue macrophages. Peritoneal granulocytes elicited in response to thioglycolate are a model of complement-mediated granulocyte recruitment. This experiment examined the hypothesis that granulocytes primed with CR3 surface-bound β -1,3-glucan could be recruited by tumors that had activated complement, thereby allowing them to recognize and kill tumor cells coated with iC3b. Elicited peritoneal granulocytes were isolated from WT and CR3-deficient mice that had been given oral β -1,3-glucan and tested for their ability to kill iC3b-coated tumor cells *in vitro*. Granulocytes from mice that had not been given oral WGP β -1,3-glucan served as a control for the

ability of non-glucan-exposed granulocytes to kill iC3b-coated tumor cells (Fig. 8). The data indicated that WT granulocytes with CR3-bound β -1,3-glucan could kill significantly more iC3b tumor cells than WT granulocytes from mice not given oral β -1,3-glucan. The requirement for CR3 was confirmed by the significantly reduced tumor-killing activity by granulocytes from CR3-deficient mice that had been given oral β -1,3-glucan. The relatively low level of cytotoxicity elicited with WT granulocytes (12%) appeared to correspond to the low proportion of peritoneal granulocytes that exhibited CR3 surface-bound β -1,3-glucan-F, i.e., ~16% (Fig. 7). This hypothesis was confirmed by showing that the cytotoxicity of elicited WT, but not CR3-deficient granulocytes, could be significantly enhanced by the addition of exogenous soluble yeast β -1,3-glucan (Fig. 8).

To determine whether the soluble β -1,3-glucan secreted by macrophages similarly mediated CR3-dependent granulocyte cytotoxicity, a BioReactor system with J774 cell-coated microcarrier beads was used to generate soluble β -1,3;1,6-glucan derived from ingested WGP. Cytotoxicity assays indicated that this macrophage-derived β -1,3-glucan produced maximum levels of cytotoxicity when used at a hexose concentration of 1.0 μ g/ml (Fig. 8). In comparison to the only currently available preparation of NSG, the macrophage-derived β -1,3-glucan when used at a concentration of 0.5 μ g/ml elicited approximately the same level of cytotoxicity as did 10 μ g/ml NSG β -1,3-glucan (not shown). As expected, the

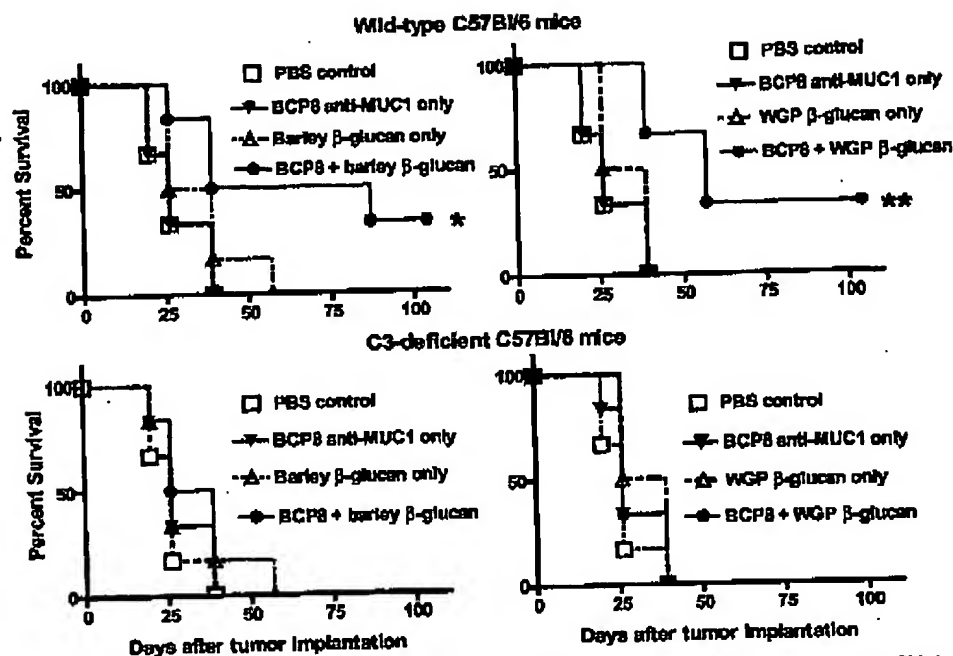


FIGURE 4. Long-term survival with orally administered soluble barley or particulate yeast β -1,3-glucan requires serum C3. This is the survival analysis of the experiment described in Fig. 4. Both the barley and yeast WGP β -1,3-glucans given in combination with mAb produced significantly greater survival than did therapy with mAb alone (**, $p < 0.01$; *, $p < 0.05$).

macrophage-derived β -1,3-glucan elicited significantly less cytotoxicity ($p < 0.0001$) with CR3-deficient granulocytes than it did with WT granulocytes (Fig. 8).

Additional experiments demonstrated that splenic macrophages from mice given oral barley β -1,3-glucan daily acquired the ability to kill iC3b tumor cells after 3 days, and that all tumor-killing activity was inhibited completely by an anti-CR3 mAb (data not shown). In conclusion, these experiments show that bone marrow granulocytes and tissue macrophages acquire membrane CR3-bound soluble β -1,3-glucan from gastrointestinal macrophages, and that this bound β -1,3-glucan primes the CR3 of both granulocytes and macrophages so that, when they are recruited to a site of inflammation, they are able to kill iC3b-coated tumor cells.

Discussion

β -1,3-Glucan functions as a potent adjuvant for mAb therapy of cancer to elicit a novel granulocyte- and tissue macrophage-mediated tumor-killing mechanism that is not activated by mAb therapy alone. Various tumor models were described in this and previous reports in which specific mAb given alone had little or no effect on tumor regression and yet mediated complete remission when given together with either oral or i.v. β -1,3-glucan (16, 17, 20, 21). This study showed that oral and i.v. β -1,3-glucans function by a similar mechanism. Although i.v. soluble NSG β -1,3-glucan is delivered directly to the CR3 on circulating granulocytes, orally administered β -1,3-glucan goes through an intermediate step in which macrophages process and deliver soluble β -1,3-glucan to the CR3 of granulocytes in the bone marrow. In addition to priming granulocyte CR3, oral β -1,3-glucan primes the CR3 of tissue macrophages and probably also the CR3 of marrow monocytes and NK cells, although these other marrow leukocyte types were not examined.

A variety of fungal and yeast (1,3;1,6-linked D-glucose) and cereal grain (1,3;1,4-linked D-glucose) β -1,3-glucans have been

reported to have antitumor activity. Most animal experimentation and clinical trials have tested fungal β -1,3-glucan such as lentinan or schizophyllan given i.v. (26–28). Notably, there have also been reports that some mushroom β -1,3-glucans functioned against cancer when given orally (18, 28–31). The current investigation showed that oral uptake and biodistribution of barley or yeast β -1,3-glucan occurred via gastrointestinal macrophages.

Current and previously reported data (16, 17, 20) show that β -1,3-glucan-mediated tumor regression requires antitumor Abs that activate complement and deposit iC3b on the tumor cells. Tumor regression elicited with β -1,3-glucan alone has been shown to be due to naturally elicited antitumor Abs that function similarly to exogenous antitumor mAb by coating tumors with iC3b. The absence of antitumor Ab in some animal tumor models, and particularly in some cancer patients, explains the inconsistent responses observed with β -1,3-glucan monotherapy. In addition to functioning with antitumor mAb, vaccines that elicit antitumor Abs are potentiated by β -1,3-glucan. Notably, a MUC1 peptide vaccine that elicited a strong Ab response but no protection from challenge with a MUC1⁺ tumor (32) was completely protective when the tumor challenge was conducted in MUC1-immunized mice given oral β -1,3-glucan (G. D. Ross, unpublished observation). Moreover, a different vaccine formulation that elicited primarily a non-complement-activating IgG1 response was not protective when combined with β -1,3-glucan.

Four humanized antitumor mAb, Herceptin (Trastuzumab, specific for Her2/neu) (33, 34), Rituxan (Rituximab, specific for CD20) (35, 36), Campath-1H (Alemtuzumab, specific for CD52) (37, 38), and Erbitux (Cetuximab, specific for Her1/EGFR) (39, 40), are now being used to treat patients with metastatic breast carcinoma, non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and metastatic colon carcinoma, respectively. Rituxan kills tumors by several mechanisms including Ab-dependent cellular

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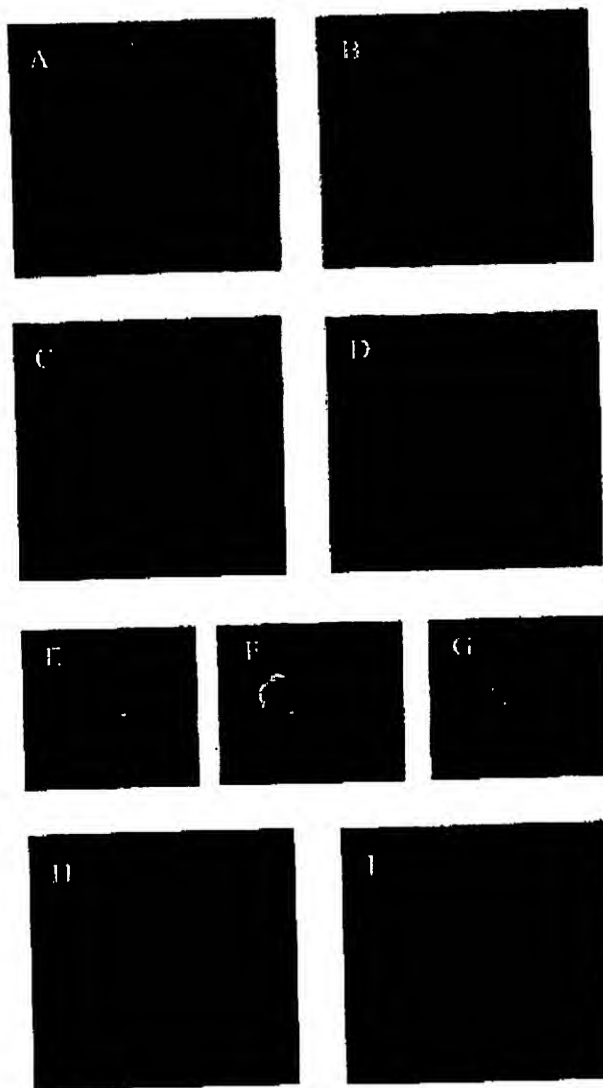
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FIGURE 5. Orally administered WGP-F or microaggregates of BG-F are taken up by macrophages that migrate to the spleen, lymph nodes, and bone marrow. As described in *Materials and Methods*, WT or CR3-deficient C57BL/6 mice were given either WGP-F or BG-F daily by oral gavage, and then groups of three mice were sacrificed at timed intervals for analysis of the lymphoid organ distribution of cells containing WGP-F or BG-F by fluorescence microscopy. *A* and *B*, Splenic macrophages isolated from WT mice 3 days after daily oral administration of WGP-F. *C* and *D*, WT (*C*) and CR3-deficient (*D*) bone marrow macrophages isolated by absorption and elution from fibronectin-coated dishes after 7 days of continuous daily oral administration of WGP-F. *E* and *F*, Bone marrow macrophage double-staining with F4/80-PE-Cy5.5 (red) confirmed that all cells containing ingested WGP-F were macrophages. *G*, From days 5 to 12 after daily oral administration of WGP-F, WT but not CR3-deficient bone marrow granulocytes exhibited membrane surface fluorescein staining. *H* and *I*, F4/80-PE-Cy5.5-stained splenic (*H*) and bone marrow macrophages (*I*) from mice fed BG-F for 7 days.

cytotoxicity and complement-dependent cytotoxicity, and can induce major clinical responses. Nevertheless, when Rituxan was given in combination with oral β -1,3-glucan in a murine xenograft model, tumor regression was significantly enhanced (20). Hercep-

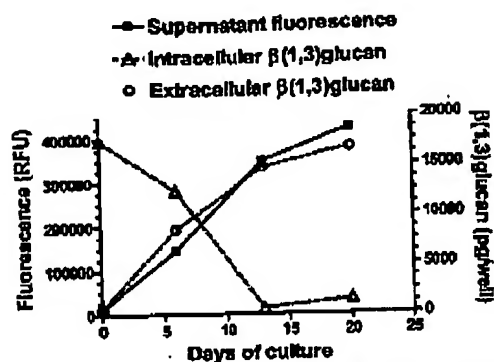


FIGURE 6. Kinetics of in vitro macrophage degradation of phagocytosed WGP-F. WGP-F was added to cultures of 1774 macrophages in six-well plates, and phagocytosis was allowed to proceed for 1 h before removal of unbound WGP-F. The amount of WGP-F remaining within macrophages was evaluated during cultivation for 3 wk by fluorescence microscopy. Harvested culture supernatants and cell lysates were sampled at the indicated intervals and tested for both fluorescein-specific relative fluorescence units (RFU) and bioactive β -1,3-glucan using the Glucastell assay.

tin and Erbitux have demonstrated only modest (<20%) responses when used as monotherapy. Both mAb function only against tumor cells that overexpress the Her2 or Her1 growth factor receptors, respectively, and only if the patient's tumor relies entirely on the growth factor receptor for survival. Both Herceptin and Erbitux contain the human IgG1 Fc region to facilitate complement activation with deposition of iC3b on tumor cells bearing the target Her2 or Her1 Ags (41, 42). In mouse xenograft models, although there was no tumor regression response mediated by Herceptin or Erbitux alone, significant regression was observed when the mAb were given in combination with oral β -1,3-glucan (20).

This investigation showed that oral β -1,3-glucan therapy was likely dependent on two types of β -1,3-glucan receptors. First, the uptake of barley and yeast WGP β -1,3-glucan by gastrointestinal macrophages occurred with CR3-deficient mice, suggesting the likely involvement of Dectin-1 (2, 9). Second, bone marrow granulocytes used CR3 to take up the soluble β -1,3-glucan released by macrophages that had partially degraded either barley or yeast WGP β -1,3-glucan. The finding of CR3 surface-bound β -1,3-glucan on isolated peritoneal granulocytes represents the first demonstration that membrane CR3 serves as a receptor for β -1,3-glucan

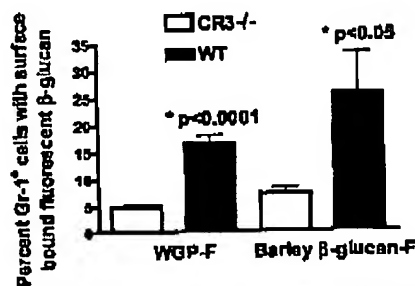


FIGURE 7. Fluorescein-labeled soluble yeast or barley β -1,3-glucans shed from bone marrow macrophages are taken up by the membrane CR3 of the margined pool of granulocytes in the bone marrow. Ten WT and 10 CR3-deficient C57BL/6 mice were given daily oral doses of WGP-F or BG-F for 10 days, and then peritoneal granulocytes were elicited with thioglycolate and analyzed for surface fluorescein staining by flow cytometry using anti-Gr-1-PE to identify granulocytes.

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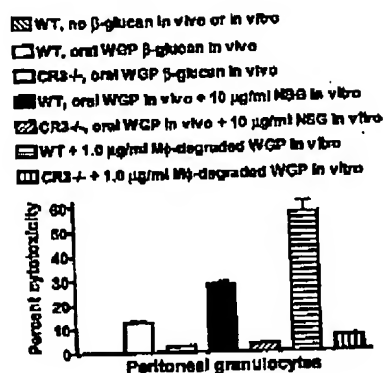


FIGURE 8. Granulocytes from WT mice that had been given daily oral doses of yeast WGP β -1,3-glucan for 10 days were able to kill iC3b-opsonized tumor cells significantly better than were granulocytes from CR3-deficient mice given the same oral doses of WGP β -1,3-glucan. Peritoneal granulocytes were isolated from WT or CR3-deficient mice that had received 10 daily oral doses of WGP β -1,3-glucan and tested for their ability mediate cytotoxicity of ^{51}Cr -labeled RMA-S-MUC1 tumor cells coated with iC3b. Data obtained with a 3:1 granulocyte-to-tumor cell ratio are shown. Granulocytes from WT mice that had not received oral β -1,3-glucan served as a control for the effect of the oral yeast WGP. To determine whether granulocytes had been fully primed by soluble β -1,3-glucan in vivo, granulocytes from both WT and CR3^{-/-} mice that had been exposed to β -1,3-glucan in vivo through orally administered WGP β -1,3-glucan were additionally treated with 10 $\mu\text{g}/\text{ml}$ soluble NSG β -1,3-glucan in vitro. Granulocytes from WT mice that had been primed in vivo by oral WGP β -1,3-glucan exhibited significantly more cytotoxicity ($p < 0.01$) than did granulocytes from either CR3^{-/-} mice that had similarly been administered oral WGP β -1,3-glucan or WT mice that had not been exposed to β -1,3-glucan in vivo or in vitro. With granulocytes from WT mice that had been primed in vivo with orally administered WGP β -1,3-glucan, there was a significant increase in cytotoxicity produced by treating the granulocytes in vitro with NSG β -1,3-glucan ($p < 0.0001$). By contrast, there was no significant increase in cytotoxicity when granulocytes from CR3^{-/-} mice primed in vivo with oral β -1,3-glucan were secondarily primed in vitro with NSG β -1,3-glucan. Soluble β -1,3-glucan secreted by cultured J774 macrophages that had ingested WGP β -1,3-glucan was 10–20 times more active on a weight basis in inducing WT granulocyte cytotoxicity than was this preparation of NSG β -1,3-glucan, but elicited only minimal cytotoxicity from CR3^{-/-} granulocytes. The data shown represent the mean values and 1 SD from three experiments conducted in triplicate.

in vivo. Although macrophages and granulocytes may also use Dectin-1 to capture soluble β -1,3-glucan, only CR3 with bound β -1,3-glucan triggers cytotoxic degranulation in response to iC3b-coated tumors (3, 14, 15). Splenic macrophages from mice given oral barley β -1,3-glucan also used CR3 to kill iC3b-tumor cells. The requirement for CR3 on leukocytes and iC3b on tumors was highlighted by demonstration of therapy failures in mice deficient in either CR3 or C3. Nevertheless, there was a low level of β -1,3-glucan-mediated tumor regression and survival in CR3^{-/-} mice treated with oral WGP β -1,3-glucan (but not barley β -1,3-glucan) that suggests a minor role for non-CR3 β -1,3-glucan receptors in tumoricidal activity. The reduced level of tumoricidal activity observed with granulocytes from CR3^{-/-} mice given oral WGP β -1,3-glucan or treated in vitro with soluble β -1,3-glucan (Fig. 8) may also be due to a non-CR3-dependent pathway of cytotoxicity.

Analysis of peritoneal granulocytes from mice that had received oral fluorescein-labeled barley or yeast WGP β -1,3-glucan showed that 16–24% of cells bore surface-bound β -1,3-glucan-fluorescein. Tests of these granulocytes for cytotoxicity of iC3b-tumor cells

revealed a low-but-significant level of CR3-dependent cytotoxicity compared with granulocytes from mice that had not been given β -1,3-glucan. Because soluble β -1,3-glucan is reversibly bound to CR3 (4), it is likely that, over the 4 h during which granulocytes were elicited to the peritoneal cavity, some of the CR3-bound β -1,3-glucan-fluorescein may have dissociated from the cell surface. Addition of soluble β -1,3-glucan to elicited peritoneal granulocytes in vitro generated a significant enhancement in their CR3-dependent killing of iC3b-tumor cells, confirming that the initial lower level of CR3-dependent tumoricidal activity was due to a partial priming of CR3 with β -1,3-glucan in vivo. Despite what appears to be minimal cytotoxicity mediated by elicited granulocytes in vitro, the essential role of granulocytes in oral, as well as i.v. (17), β -1,3-glucan therapy was confirmed by showing complete inhibition of tumor regression activity in mice depleted of granulocytes with anti-Gr-1 mAb (G. D. Ross, unpublished data). Thus, even though the CR3 of macrophages are primed in vivo with β -1,3-glucan, the tumoricidal activity of macrophages is insufficient to mediate tumor regression without granulocytes. The previous demonstration that antitumor mAb plus β -1,3-glucan is equally effective in SCID or nude mice as compared with WT mice, also indicates that this mechanism of tumor rejection does not require T cells (16, 20, 21). Depletion of NK cells also had no effect on tumor regression (21). Nevertheless, the predominant role of granulocytes has been demonstrated only with s.c. tumors, and thus it remains possible that tumors in other anatomic locations may be more effectively killed by macrophages. For example, the gastrointestinal macrophages that have taken up oral β -1,3-glucan may be more effective against nearby gastrointestinal tumors than against distant s.c. tumors that granulocytes may be able to reach more quickly via the blood.

This research provides the basis for clinical trials that have recently been initiated in which patients are treated with antitumor mAb and oral β -1,3-glucan. Therapy with mAb or vaccines in combination with β -1,3-glucan offers several advantages over immunotherapy protocols designed to elicit immune CTL. First, it is usually easier to elicit an Ab response than a CTL response with a tumor vaccine. Second, the choice of tumor Ags includes carbohydrates and gangliosides, and is not limited to specific peptide sequences that can be presented by MHC class I molecules. Third, metastatic tumors cannot escape immune recognition by down-regulating their expression of MHC class I as occurs frequently with CTL responses (43–45). Fourth, β -1,3-glucan therapy can use humanized mAb to target tumors and thus does not rely on the patient's own immune response, which is frequently suppressed because of tumor burden and/or previous chemoradiation therapy. Nevertheless, the use of antitumor mAb and β -1,3-glucan should not interfere with the function of CTL-based immunotherapy, and the targeting of tumors simultaneously with both CTL and granulocytes should provide a more effective means of eliminating tumors and developing a long-term tumor-specific immunity that prevents tumor recurrence.

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